OSMOREGULATION IN A MARINE CILIATE, *MIAMIENSIS AVIDUS*. II. REGULATION OF INTRACELLUAR FREE AMINO ACIDS [1]

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Some aspects of osmotic and ionic regulation in *Miamiensis avidus,* a marine ciliate, are known (Kaneshiro, Dunham and Holz, 1969). As in freshwater protozoa, the contractile vacuole is involved in regulation of cell volume. This was shown by studies on the relationship between external osmolarity and the rate of vacuolar output. Intracellular Na^+ and Cl^- are maintained lower than the external concentrations in a wide range of external salinities, but the intracellular concentrations of these ions change more or less proportionally with changes in the external concentrations. Potassium, as well as Ca^{++} and Mg^{++}, are maintained relatively constant despite large changes in the external concentrations. Although not all of the intracellular osmotic constituents were identified, the evidence suggested that *Miamiensis avidus* maintains itself hyperosmotic to the environment over a wide range of external salinities.

Information on osmoregulation in other marine protozoa is very limited. It comes primarily from studies of vacuolar function (see Kitching, 1967, for references). Measurements of the inorganic ionic composition of *Uronema filificum* have also been made (Kaneshiro *et al.,* 1969).

The concentrations of free amino acids in cells of marine invertebrates are generally large, and apparently serve an osmoregulatory function (Potts and Parry, 1964). We have analyzed the free amino acids in *Miamiensis avidus,* and have demonstrated that regulation of intracellular free amino acids is involved in osmoregulation in this marine ciliate.

MATERIALS AND METHODS

Environmental osmolarity variation and ion substitutions

Mass cultures of *Miamiensis avidus* were grown as described in Kaneshiro *et al.* (1969). Appropriate concentrations of artificial sea water (*Marine Biological Laboratory Formulae and Methods V,* 1964), were added to cell suspensions to produce high osmolarity media. Distilled water was added for low osmolarity media. In some experiments sucrose (0.72 M) isosmotic to 31‰ salinity sea water was

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added to maintain osmotic pressure when inorganic ion concentrations were varied. All final equilibration media, except 25% sea water, were prepared by adding equal amounts of cells suspended in the culture medium and distilled water or high osmolarity solutions. The 25% sea water test medium was prepared by adding three times the amount of distilled water to the cell suspension. In individual ion substitution experiments, Na^+, K^+ and Ca^{++} were replaced by choline$^+$, Mg^{++} was replaced by Na^+, and Cl^- was replaced by a combination of propionate$^-$ and SO_4^{--}.

Amino acid analysis

Miamiensis avidus, collected by centrifugation after the above experiments, was prepared for analysis as described by Kaneshiro *et al.* (1969). Norleucine (Technicon Chemical Co., Chauncey, New York; 2.5 μ moles/ml) was added to packed cells as an internal standard.

The intracellular free amino acids were extracted with 70% ethanol (10 ml, 5° C, 24 hr). The cell debris was then centrifuged and washed by centrifugation with 5 ml, then 2 ml, 70% ethanol. Boiling the pellet in distilled water after the ethanol extraction did not increase the yield of amino acids. The three ethanol supernatants were pooled and added to 6× their pooled volume of chloroform, and the mixture was shaken and allowed to stand for 12 hours at 5° C. The aqueous phase was removed and the chloroform phase washed with 5 ml, then 2 ml, 0.01 N HCl. The washes were pooled with the aqueous phase and the total taken to near dryness with a rotary vacuum evaporator. The sample was then redissolved in 0.01 N HCl (for thin layer chromatography and Technicon automatic amino acid analysis) or pH 2.2 Na-citrate buffer (for Beckman automatic amino acid analysis) and filtered (Millipore, 0.45 μ pore size).

Extracellular amino acids from experimental media were separated from the salts by passing a 4-ml aliquot through a column of ion retardation resin (Bio-Rad, Richmond, Calif., AG 11A8, 50–100 mesh). Distilled, deionized water was the eluant and regenerant. Ninhydrin was used for detecting amino acids and silver nitrate for detecting chloride salts in the eluate fractions.

Identification of alanine, glycine and proline was done by cochromatography with standards in two systems: 1. Two-dimensional thin layer chromatography: Eastman Chromatogram, silica gel sheet, type K 301 R2 was the support and stationary phase. n-Butanol:glacial acetic acid:water (80:20:20) and chloroform:methanol:17% ammonium hydroxide (40:40:20) were the solvent systems. Ninhydrin was the indicator. 2. Ion exchange column chromatography:Enhancement of peaks of standards on automatic amino acid analyzer recording tracings was accepted as evidence of cochromatography.

Other amino acids were provisionally identified by method 2 only.

Concentrations of individual amino acids were determined by comparisons of peak areas of amino acid analyzer recorder tracings with that of the norleucine standard. Unidentified peaks were estimated by using the leucine peak area as a reference. Values obtained for the free amino acid content of cells were corrected for inulin space of packed pellets and for cell volume changes (Kaneshiro *et al.,* 1969).
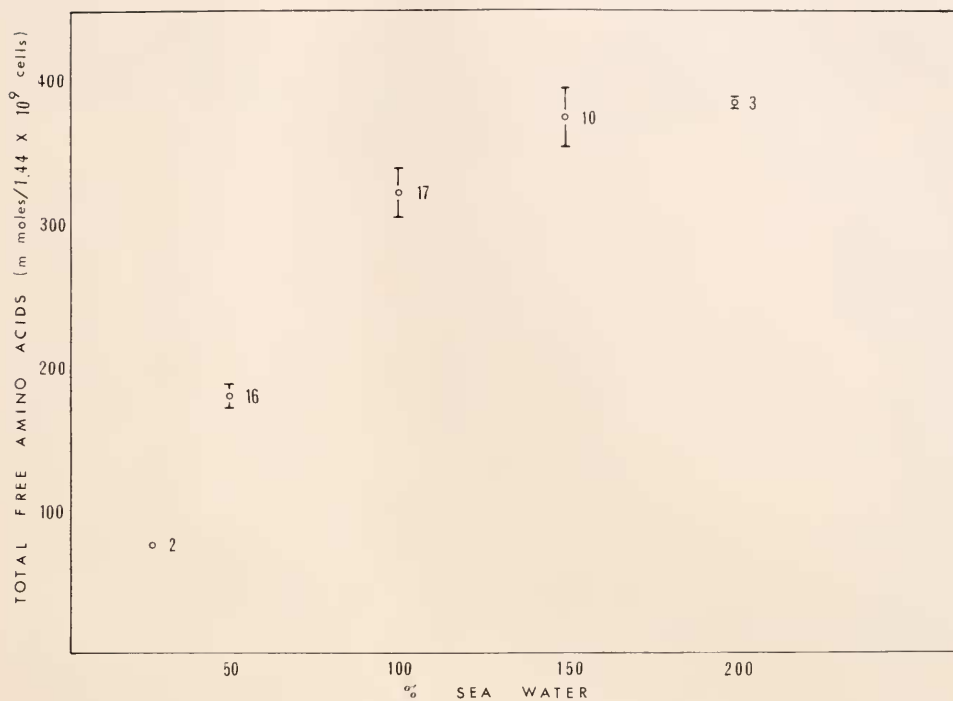
FIGURE 1. Total intracellular free amino acid content in *M. avidus* after equilibration (20–90 minutes) in media of various salinities. Since cell volumes changed under the various experimental conditions, the contents are expressed as mM/1.44 × 10^9 cells, the number of cells in 100% sea water comprising a liter. Means, standard errors of the means, and numbers of determinations are indicated.

Amino acid uptake

To determine if extracellular amino acids contributed to intracellular free amino acid pools during exposures (5–60 min) to a variety of salinities (from 25 to 200% sea water), cells were incubated with 10 mM L-alanine per liter of the various concentrations of sea water and sufficient U-C^{14}-L-alanine (Calbiochem, sp. act. −34.4 mC/mM) to give a final radioactivity of 0.02 mC/mM (4.4 × 10^5 dpm/ml) in each incubate. Radioactivities of 1-ml samples of ethanol extracts of cells, prepared as above, ethanol-insoluble cell debris (solubilized with Hyamine 10-X) and experimental test solutions were determined in a liquid scintillation spectrometer. H^3-inulin (New England Nuclear) was used for inulin space corrections (Dunham and Child, 1961).

Amino acid uptake was also tested with a mixture of U-C^{14}-L-amino acids derived from hydrolysis of a uniformly labelled algal protein (Volk Radiochemicals, Chicago, Illinois, sp. act.—0.8 mC/mg). The final radioactivity of the various concentrations of sea water was 10,000 dpm/ml. In these experiments, ethanol extracts of cells and samples of media were dried on stainless steel planchets and counted with a thin-window gas flow detector.

TABLE 1

The amounts of individual free amino acids in M. avidus *equilibrated for* 20–90 *minutes in media of various salinities.* The amino acids are expressed as mM/1.44 $\times$ 10^9 cells, the number of cells comprising a liter in 100% sea water. The salinities are given as per cent of sea water. Numbers of determination of each amino acid at each salinity are given in parentheses

Amino acid	25(2)	50(16)	100(17)	150(10)	200(3)
Aspartic acid	0.3	1.9	1.7	4.6	1.4
Threonine	1.1	6.6	8.7	12.6	30.0
Serine + asparagine	6.4	11.8	19.3	22.6	15.6
Glutamic acid	2.8	7.7	7.8	11.0	6.1
Proline	8.1	20.9	45.6	44.9	75.3
Glycine	15.4	35.1	64.8	65.8	76.7
Alanine	27.7	66.8	128.6	154.6	142.0
Cystine	0.9	1.4	1.3	1.9	—
Valine	2.9	6.1	10.0	13.4	8.4
Methionine	0.5	0.9	1.6	2.5	0.6
Isoleucine	1.0	2.3	3.2	5.8	2.5
Leucine	1.9	4.2	6.7	10.5	4.7
Tyrosine	—	0.6	1.3	1.5	0.8
Phenylalanine	0.3	1.0	1.2	2.1	1.4
Lysine	0.9	2.6	2.2	4.6	1.2
Histidine	0.3	0.7	0.8	1.3	0.8
Arginine	1.5	2.9	3.0	3.0	3.3
Unknowns (at least 10 detected)	2.9	7.4	8.8	11.5	14.3
Total	74.9	180.9	316.6	374.2	385.1

RESULTS

The total intracellular concentration of free amino acids in *M. avidus* in 100% sea water was 317 mM/kg cells. This is in the range of concentrations in most tissues of marine metazoans which have been analyzed (Potts and Parry, 1964).

The three most prevalent free amino acids in *M. avidus* were alanine > glycine > proline. They accounted for an average of 73% of the total free amino acids in 82 determinations under various conditions.

When the external salinity was decreased, the amount of free amino acids per cell decreased and when the external salinity was increased the amount of free amino acids per cell increased. Figure 1 shows the total free amino acid content in cells equilibrated in various salinities. The effect of a two-fold reduction in salinity was much greater than the effect of a two-fold increase. Table I shows the concentrations of the individual amino acids of *M. avidus* after equilibration in the different salinities. They are listed in the order of their elution from the ion exchange column; the most acidic amino acids are retarded by the resin the least and appeared in the eluate first. Most of the free amino acids in *M. avidus* were acidic. The concentration of total free amino acids in 50, 100 and 150% sea water test solutions was 9 mM/1. The concentration in the culture medium, before dilution with distilled water or high osmolarity solution was 21 mM/1. The free amino acid concentrations of ciliates in 100% sea water culture medium and 100% sea water controls (cell suspension + 100% M. B. L. formula sea water, 1:1) were the same, so the intracellular changes were not due to changes in the
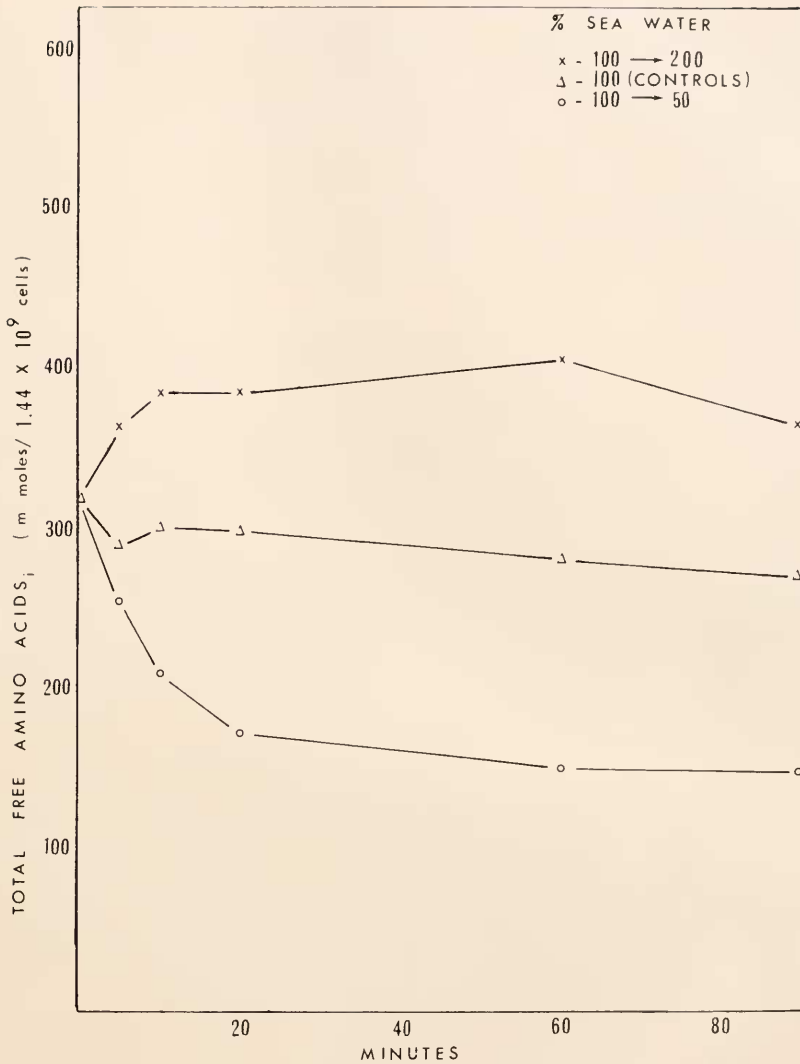
FIGURE 2. Kinetics of changes in total free amino acid content of *M. avidus* after transfer from 100% sea water to 50% or 200% sea water. Contents are expressed as mM/1.44 × 10⁹ cells, the number of cells in 100% sea water comprising a liter. Each point represents a single determination.

external amino acid concentration. As shown in Figure 2, the changes in intra-cellular free amino acid content after transfer to either higher or lower salinity were essentially complete in 20 minutes.

Taurine, the very acidic analogue of alanine, has been detected in high con-centrations in tissues of numerous marine invertebrates (Simpson, Allen and Awapara, 1959; Allen and Awapara, 1960; Awapara, 1962) but was not found in *M. avidus*.

When the external ion concentration was decreased by 50% but the external osmolarity was kept equivalent to that of sea water by addition of sucrose, the concentration of intracellular free amino acids, 285 mM/1.44 × 10⁹ cells (±37 mM, S. E.; 8 determinations), was much greater than in cells in 50% sea water (181 mM/1.44 × 10⁹ cells). It was not much lower than in cells in 100% sea water. Substitutions of choline⁺ for Na⁺, K⁺ or Ca⁺⁺, Na⁺ for Mg⁺⁺ or propionate⁻ and SO₄⁻⁻ for Cl⁻ did not affect intracellular amino acid content. The decrease in intracellular free amino acid content, therefore, is a response to decreased external osmolarity and not to decreased ionic strength or specific ion concentrations.

Amino acid uptake

In none of the experiments in which cells were exposed to C¹⁴-amino acids for up to one hour after transfer to higher or lower salinities was there measurable uptake of amino acids; neither C¹⁴-alanine nor the mixed, labelled amino acids. In 62 samples of cells taken in 4 experiments, the radioactivities could always be accounted for by the extracellular space of the packed pellets of cells.

As shown in Table I, the measured increase of free alanine in cells after transfer from 100% to 200% sea water was 14 mM/original liter of cells. If 5% of this increase were the result of accumulation from the medium, it would have been resolved easily with the specific activity of alanine and the amounts of cells used in the experiments. Therefore, uptake of alanine from the medium could account for only a small fraction of the increase in free alanine content in the cells. A similar argument can be developed for the experiments using a mixture of C¹⁴-amino acids.

DISCUSSION

The results presented here show that osmoregulation in *Miamiensis avidus* is accomplished in part by regulation of free amino acids. Increasing or decreasing external osmolarity resulted in an increase or decrease, respectively, in intracellular free amino acids. It was shown previously that intracellular Na⁺ and Cl⁻ are altered in a similar manner in response to changes in osmolarity. These changes, particularly of the amino acids, do not appear to be simply passive responses, but rather are likely to reflect regulatory processes enabling the cell to maintain a constant relationship between intracellular and external osmotic pressure.

It had been suggested previously, mainly on the basis of observations of the function of the contractile vacuole, that *M. avidus* maintained itself hyperosmotic to all environments, even in sea water concentrated more than two-fold (Kaneshiro *et al.*, 1969). Preliminary experiments on freezing point depressions of intact *M. avidus* cells have confirmed this suggestion.

The principal intracellular solutes which have been measured, Na⁺, K⁺, Cl⁻, and free amino acids, total about 540 mM/kg cells in 100% sea water, and probably do not account for all the intracellular osmolarity of *M. avidus*. However, to know their actual contribution requires knowledge of cell water content, activity coefficients, and the extent and nature of compartmentation of solutes and water. The unmeasured solutes might be such substances as orthophosphate and phosphate esters, which are major constituents in *Tetrahymena*, a fresh-water ciliate (Cline and Conner, 1966).

It is concluded that the changes in free amino acid content reflect the regulatory process which maintains the cells hyperosmotic to their environment. The functional significance of this hyperosmotic state may be to obtain water to operate the contractile vacuole. The contractile vacuole may be responsible for the elimination of some metabolic wastes. This structure may also be involved in the maintenance of the low intracellular Na^+ concentration (cf. Dunham, 1969). The elimination of these various solutes would require a certain volume of water, and the maintenance of a hyperosmotic state would provide the supply of water.

Maintenance of a hyperosmotic internal state is characteristic of fresh water and brackish water animals, but among true marine metazoans only the elasmobranchs are hyperosmotic. Probably all marine animals which maintain an osmolarity different from sea water are secondarily marine, and no marine animals without fresh-water or brackish water ancestors have evolved the ability to maintain an osmotic gradient. It has been postulated before that marine ciliates had fresh water ancestors (Potts and Parry, 1964), but no concrete evidence was available. Hyperosmotic regulation in marine ciliates constitutes evidence of fresh water ancestry. However, *Miamiensis avidus* is the only marine ciliate for which such evidence is available.

Decreases of intracellular free amino acid concentrations in response to salinity decreases have been observed in tissues of numerous metazoans. (*Carcinus* muscle—Shaw, 1958, Duchâteau, Florkin and Jeuniaux, 1959; *Mytilus* muscle— Potts, 1958; *Eriocheir* nerve—Schoffeniels, 1960; *Arenicola* muscle—Duchâteau-Bosson, Jeuniaux and Florkin, 1961; *Golfingia* body wall—Virkar, 1966; *Limulus* muscle—Bricteux-Gregoire, Duchâteau-Bosson, Jeuniaux and Florkin, 1966; *Crassostrea* muscle—Lynch and Wood, 1966; *Rana cancrivora* muscle—Gordon and Tucker, 1968). The decrease of free amino acid content in *M. avidus* and in the metazoan tissues in low salinities may be due to loss from the cells. However, Shaw (1958) observed no increase in the amino acid concentration in extracellular fluid of *Carcinus* when the crabs were acclimated to dilute sea water.

Prior observations of an increase in free amino acid content in response to increased salinity have been made for *Eriocheir* nerve and muscle of *Rana cancrivora*. Isolated nerves of *Eriocheir* adapted to 50% sea water increased their free amino acid concentration when placed in 100% sea water (Schoffeniels, 1960). Free amino acid concentrations in muscle of *Rana cancrivora* was much higher when the frog was acclimated in 80% sea water than when it was in fresh water (Gordon and Tucker, 1968).

The failure of *M. avidus* to accumulate C^{14}-amino acids shows that the mechanism of increased free amino acid content after increased salinity is mobilization of amino acids from protein or from other bound states within the cell. It may be surprising that no uptake of amino acids from the medium was observed under any conditions, considering that *Tetrahymena* rapidly accumulates C^{14}-phenylalanine (Stephens and Kerr, 1962).

It has been suggested that the decrease in amino acid concentration in several metazoan tissues placed in diluted sea water is due to polymerization of amino acids or binding rather than loss from the cells (Shaw, 1958; Schoffeniels, 1960; Stephens and Virkar, 1966). The decrease in free amino acids in *M. avidus* may be accomplished by the same means, the reverse of the indicated mechanism of

the increase. It should be mentioned in this regard that *Tetrahymena* continually loses amino acids to an inorganic medium (Crockett, Dunham and Rasmussen, 1965; Cann, 1968).

Results presented above suggest that the changes in free amino acid content in *M. avidus* are a response to changes in osmolarity and not to changes in ionic strength, concentrations of individual inorganic ions or free amino acids. Nothing is known of the mechanism by which change in osmolarity elicits the response by the cells.

Summary

1. The total free amino acid concentration in *Miamiensis avidus* in 100% sea water is 317 mM/kg cells. Alanine, glycine and proline account for 73% of the total.

2. Reducing the external osmolarity resulted in a decrease in intracellular free amino acids; raising the external osmolarity resulted in an increase in free amino acids. At the extreme salinities tested, cells in 25% sea water contained 24% of the free amino acids of cells in 100% sea water; cells in 200% sea water had a 22% greater free amino acid content than cells in 100% sea water. All of the changes were complete 20 minutes after the salinity changes.

3. The intracellular free amino acid content was a function of external osmolarity, and not ionic strength or concentration of individual ions.

4. The mechanism of the increase in free amino acid content was mobilization of bound amino acids, and not uptake from the medium.

5. These results, together with earlier findings on regulation of ions and water in *Miamiensis avidus*, suggest that this organism maintains itself hyperosmotic to its environment over a wide range of external salinities. The functional significance of the hyperosmotic state may be to obtain water to operate the contractile vacuole. The possibility of a fresh water ancestry for marine ciliates is discussed.

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